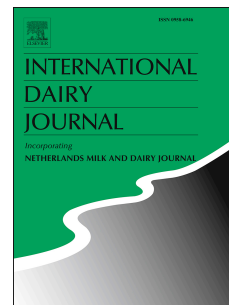


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**Immunomodulatory activity of 5 kDa permeate fractions of casein hydrolysates  
generated using a range of enzymes in Jurkat T cells and RAW264.7 macrophages**

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## ABSTRACT

The in vitro bioactivity of 5 kDa ultrafiltration permeate fractions of casein hydrolysates produced using different enzymes were compared. Reverse phase ultra-performance liquid chromatography and gel permeation chromatography showed that the permeates had different physicochemical properties (molecular mass and degree of hydrolysis). The Flavourzyme® permeate had the highest activity in the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay. Cellular antioxidant and immunomodulatory assays showed that none of the permeates exhibited in vitro antioxidant activity, while all permeates significantly ( $P < 0.05$ ) decreased interleukin-6 (IL-6) production in ConA-stimulated Jurkat T cells at 0.50% (w/v) and LPS-stimulated RAW264.7 cells at 0.05 and 0.50% (w/v). Three permeates, obtained using Flavourzyme®, Flavorpro Whey and trypsin, also significantly ( $P < 0.05$ ) decreased IL-1 $\beta$  production at 0.05% (w/v) in RAW264.7 cells. Western blot analysis showed that all permeates significantly decreased the expression of the NF- $\kappa$ B subunit, p65, in RAW264.7 cells indicating that anti-inflammatory activity may be associated with this pathway.

## 1. Introduction

Cardiovascular disease (CVD), in particular atherosclerosis, is associated with elevated inflammation and oxidative stress. Drugs used to treat these conditions may produce unwanted side effects, therefore natural alternatives to synthetic drugs are constantly sought (Chakrabarti, Jahandideh & Wu, 2014). Bioactive peptides are small protein fragments that have the potential to exert beneficial health effects in vivo (Urista, Fernández, Rodríguez, Cuenca & Jurado, 2011). Bioactive peptides, derived from the milk protein casein, demonstrate numerous bioactivities such as antihypertensive, opioid and antimicrobial activity (Di Pierro, O’Keeffe, Poyarkov, Lomolino, & FitzGerald, 2014; Kazlauskaitė et al., 2005; Nongonierma, O’Keeffe, & FitzGerald, 2016; Phelan, Aherne-Bruce, O’Sullivan, FitzGerald, & O’Brien, 2009; Tang et al., 2015; Trivedi, Zhang, Lopez-Toledano, Clarke, & Deth, 2016; Yamada et al., 2015). The enzymes used in the generation of these peptides, as well as the length and amino acid sequence of the resultant peptides influence the bioactivities observed (Power, Jakeman, & FitzGerald, 2013).

Casein hydrolysates with cellular antioxidant activity have been reported in numerous studies. Garcia-Nebot, Cilla, Alegría, and Barberá (2011) reported that caseinophosphopeptides showed cyto-protective effects against H<sub>2</sub>O<sub>2</sub>-induced oxidative stress in Caco-2 cells. Xie, Wang, Ao, and Li (2013) reported that an Alcalase® generated hydrolysate protected HepG2 cells from H<sub>2</sub>O<sub>2</sub>-induced oxidative damage. Hydrolysis of bovine casein glycomacropeptide with papain was also reported to protect against H<sub>2</sub>O<sub>2</sub>-induced oxidation in RAW264.7 cells, along with increasing the level of cellular antioxidant enzymes (Cheng, Gao, Song, Ren, & Mao, 2015). Treatment of Jurkat T cells with casein hydrolysates generated using different mammalian, plant or bacterial enzymes has previously been reported to increase cellular antioxidant levels (Lahart et al., 2011; Phelan et al., 2009).

More recently, the hydrolysis of casein using Prolyve®, generated a hydrolysate which prevented H<sub>2</sub>O<sub>2</sub>-induced DNA damage in U937 cells (Cermeño, FitzGerald, & O'Brien, 2016).

Casein hydrolysates have also been studied for their immunomodulatory and anti-inflammatory potential. The tryptic hydrolysis of casein generated a hydrolysate with immune enhancing effects in mouse macrophages (Kazlauskaite et al., 2005), while  $\beta$ -casein and several peptides within  $\beta$ -casein have been reported to decrease proliferation in murine spleen cells (Bonomi et al., 2011). Studies have also reported that the activity of NF- $\kappa$ B, one of the major inflammatory signalling pathways, may be reduced in cells exposed to casein hydrolysates (Altmann et al., 2016; Malinowski, Klempt, Clawin-Rädecker, Lorenzen, & Meisel, 2014). The hydrolysis of casein using mammalian, bacterial or plant derived enzymes has also been reported to have immunomodulating effects on Jurkat T cells in other studies (Cermeño et al., 2016; Lahart et al., 2011; Phelan et al., 2009). To the best of our knowledge, no study has compared the cellular antioxidant and anti-inflammatory activities of sodium caseinate hydrolysates generated under the same conditions [hydrolysis time and enzyme:substrate (E:S) ratio], using a range of proteolytic preparations.

The aims of the present study were: (i) to generate and examine the physicochemical characteristics of 5 kDa permeates of casein hydrolysates generated using seven different proteolytic preparations, (ii) to compare the antioxidant activity of the hydrolysates in vitro and in H<sub>2</sub>O<sub>2</sub>-challenged U937 cells; (iii) to determine cytotoxicity in RAW264.7 mouse macrophages, Jurkat T cells and U937 lymphocytes and (iv) to compare the immunomodulatory activity of the 5 kDa permeates of the hydrolysates in RAW264.7 macrophages and Jurkat T cells.

## 2. Materials and methods

## 2.1. Materials

Human Jurkat T cells, RAW264.7 mouse macrophages and human U937 lymphocytes were purchased from the European Collection of Animal Cell cultures (Salisbury, UK). Foetal bovine serum (FBS) was purchased from Invitrogen (Paisley, Scotland, UK). Cell culture plastics were supplied by Cruinn Diagnostics (Dublin, Ireland). Sodium caseinate (87.57% (w/w) protein) was from Arrabawn Co-op (Tipperary, Ireland). Protease from *Bacillus licheniformis* (Alcalase® 2.4L), protease from *Aspergillus oryzae* (Flavourzyme®, ≥ 500 U g<sup>-1</sup>) and TPCK-trypsin were from Sigma-Aldrich (Wicklow, Ireland). Prolyve 1000 was from Lyven Enzymes Industrielles (Caen, France) and Flavorpro Whey, Promod 144MG and Pepsin were all from Biocatalysts (Cefn Coed, Wales, UK). All other cell culture reagents and chemicals including concanavalin A (ConA), lipopolysaccharide (LPS) and Trolox were purchased from Sigma-Aldrich, unless otherwise stated.

## 2.2. Generation of casein hydrolysates, determination of the degree of hydrolysis and ultrafiltration

Sodium caseinate, reconstituted at 10% (w/v) in distilled water, was equilibrated at 50 °C with gentle mixing for 1.5 h and the pH was adjusted to pH 7 (or pH 2 for peptic hydrolysis). Enzymatic hydrolysis was carried out using a pH Stat (718 Stat Titrino, Metrohm, Herisau, Switzerland) as previously described (Spellman, McEvoy, O’Cuinn, & FitzGerald, 2003). Hydrolysis was performed using seven different proteolytic preparations (Alcalase® 2.4L, Prolyve 1000, Flavourzyme®, Flavorpro Whey, Pepsin, TPCK-Trypsin and Promod 144 MG) at industrially relevant E:S ratios (0.23% for Alcalase® 2.4L, Prolyve 1000

and Flavourzyme®, Flavorpro Whey and Promod 144 MG, 0.13% for trypsin and 0.25% for pepsin) for 4 h, except for the tryptic hydrolysate that was generated over 3 h.

Following hydrolysis, the enzymes were inactivated by heating at 80 °C for 20 min. Control samples were also included; (i) sodium caseinate was incubated at 50 °C without enzyme and (ii) enzyme was incubated at 50 °C without sodium caseinate. These control samples were also subjected to heating at 80 °C for 20 min after 4 h incubation. All samples were freeze-dried (FreeZone 18L, Labconco, Kansas City, USA) and stored at -20 °C until use. The degree of hydrolysis (DH) of the hydrolysates was determined using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method of Adler-Nissen (1979) and as described by Le Maux, Nongonierma, Barre, and FitzGerald (2016). Ultrafiltration (UF) fractions were generated by passing the hydrolysate through a membrane having a nominal cut-off of 5 kDa using a benchtop ultrafiltration system (Sartoflow Alpha, Sartorius AG, Goettingen, Germany) as described by O'Keeffe and FitzGerald (2014) and were freeze-dried as above.

### 2.3. Reverse phase ultra-performance liquid chromatography and gel permeation high performance liquid chromatography analysis of UF permeates of casein hydrolysates

Freeze-dried hydrolysates/ultrafiltration permeates were reconstituted at 1 mg mL<sup>-1</sup> in mobile phase A [0.1% trifluoroacetic acid (TFA) in MS grade H<sub>2</sub>O] and 7 µL was separated on an ACQUITY ultra-performance liquid chromatography (UPLC) system (Waters, Milford, Massachusetts, USA) at a flow rate of 0.2 µL min<sup>-1</sup> using an ACQUITY BEH 300 C18 column (2.1 × 50 mm, 1.7 µm; Waters, Dublin, Ireland). Mobile phase B was 0.1% TFA in 80% ACN. Separation was achieved using a linear gradient; 0–0.28 min 100% A; 0.28–45 min 100–20% A; 45–46 min 20–0% A; 46–48 min 0% A; 48–49 min 0–100% A; 49–51 min 100% A. Detector response was measured at 214 nm. Gel permeation high performance



liquid chromatography (GP-HPLC) was performed as previously described (Spellman, O’Cuinn, & FitzGerald, 2009) with separation achieved through isocratic elution (mobile phase: 0.1% TFA in 30% ACN at 1.0 mL min<sup>-1</sup>) on a TSK G2000 SW column (600 × 7.5 mm ID) connected to a TSKGEL SW guard column (75 × 7.5 mm ID) and the eluent was monitored at 214 nm.

#### 2.4. 2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) assay

The 2,2’-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)<sup>+</sup> (ABTS<sup>+</sup>) radical scavenging assay was carried out as described by Re et al. (1999). The ABTS<sup>+</sup> radical was prepared by incubating ABTS solution (7 mM) with potassium persulfate (2.45 mM), an oxidizing agent, in a ratio of 1.0:0.5 (v/v) at 20 °C for 16 h in the dark. The radical was then diluted using phosphate buffered saline (5 mM, pH 7.4) until an absorbance of 0.70 ± 0.02 at a wavelength of 734 nm was achieved. Activity was reported based on a standard curve using Trolox and expressed as µmol Trolox equivalents per gram of freeze-dried powder of hydrolysate (FDP). The scavenging activity for each sample was determined by three independent experiments.

#### 2.5. Oxygen radical absorbance capacity assay

The oxygen radical absorbance capacity (ORAC) assay was performed according to the method of Zulueta, Esteve, and Frígola (2009) with modifications as described by O’Keeffe and FitzGerald (2014). The final ORAC values were expressed as µmol of Trolox equivalents per mg of FDP and were the mean ± the standard error of three independent determinations.

## 2.6. Cell culture

Jurkat T and U937 cells were maintained in Royal Park Memorial Institute (RPMI) medium supplemented with 10% (v/v) foetal bovine serum (FBS). RAW264.7 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) FBS. All cell lines were cultured in an atmosphere of CO<sub>2</sub>-air (5:95, v/v) at 37 °C and were maintained in the absence of antibiotics. The dried 5 kDa permeates of the casein hydrolysates were solubilised to a concentration of 10% (w/v) using distilled deionised water, sterile-filtered using a low protein binding 0.22 µm Durapore<sup>TM</sup> millex filter unit (Merck KGaA) and diluted with sterile DMEM.

## 2.7. Cell proliferation assay

Jurkat T, U937 and RAW264.7 cells were exposed to increasing concentrations of the different 5 kDa permeates (0.05 to 5.0%, w/v) for 24 h in 96 well plates in a final volume of 200 µL. Cell proliferation was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay (MTT I proliferation kit, Roche Diagnostics; Burgess Hill, West Sussex, UK).

## 2.8. Alkaline single cell gel electrophoresis (comet) assay

U937 cells were used to assess the DNA protective effects of the 5 kDa permeates in oxidant challenged cells. Cells were seeded at a density of  $1 \times 10^5$  cells mL<sup>-1</sup> and exposed to the permeates (0.05%, w/v) for 24 h. DNA damage was then initiated by exposing cells to 80

191  $\mu\text{mol L}^{-1}$   $\text{H}_2\text{O}_2$  for 30 min at 37 °C after which DNA damage was assessed using the comet  
192 assay as previously described (Phelan et al., 2009).

### 194 2.9. Cytokine production in Jurkat T and RAW264.7 cells

195  
196 Jurkat T cells were seeded at a density of  $2 \times 10^5$  cells  $\text{mL}^{-1}$  and simultaneously  
197 incubated with ConA ( $25\mu\text{g mL}^{-1}$ ) and the 5 kDa permeates (0.50 and 0.05%, w/v) for 24 h.  
198 Following incubation, the quantity of interleukin (IL)-6, interferon (IFN)- $\gamma$ , IL-2 and IL-10  
199 in the media was measured by enzyme-linked immunosorbent assay (ELISA) (eBioscience,  
200 Insight Biotechnology Ltd, Wembley, UK). RAW264.7 cells were seeded at a density of  $0.2$   
201  $\times 10^5$  cells  $\text{mL}^{-1}$  and simultaneously incubated with LPS ( $0.1\mu\text{g mL}^{-1}$  for IL-6 and tumour  
202 necrosis factor (TNF)- $\alpha$ ;  $2\mu\text{g mL}^{-1}$  for IL-1 $\beta$ ) and the 5 kDa permeates (0.050% and 0.005,  
203 w/v) for 24 h. Following incubation, the content of each of the three cytokines (IL-6, IL-1 $\beta$   
204 and TNF- $\alpha$ ) in the media was measured using ELISA kits. Absorbance was determined at  
205 450 nm with a reference wavelength of 570 nm (Varioskan™ Flash Multimode Reader,  
206 Thermoscientific, Waltham, MA, USA).

### 208 2.10. Western blotting

209  
210 RAW264.7 cells were seeded at a density of  $8 \times 10^5$  cells per dish in 60 mm dishes  
211 and allowed to adhere overnight. Cells were then stimulated using LPS ( $0.1\mu\text{g mL}^{-1}$ ) and  
212 treated with the permeates (0.05%, w/v) for 24 h. After this treatment, the RAW264.7 cells  
213 were washed using ice cold PBS and lysed using RIPA buffer containing protease (Halt  
214 protease inhibitor, Sigma 78439) and phosphatase inhibitors (1 mM  $\text{NaVO}_4$ , 2.5 mM  $\text{Na}_4\text{O}_7\text{P}_2$   
215 and 2 mM  $\beta$ -glycerophosphate). Cell lysates were then scraped and transferred to Eppendorf

tubes and placed on ice for 20 min. Lysates were centrifuged at  $25,155 \times g$  for 1 h at 4 °C and the supernatant transferred to fresh Eppendorf tubes. Protein concentration was determined using the BCA method (Smith et al., 1985) and samples were stored at –80 °C until Western blot analysis.

Proteins were separated using a 10% sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) gel and transferred to a polyvinylidene fluoride membrane. The membrane was then blocked overnight at 4 °C using Odyssey Blocking buffer with 0.1% Tween-20. The following day, the membrane was washed using Tris-buffered saline-Tween-20 (TBS-T) and incubated with mouse anti-p65 antibody overnight at 4 °C. Following incubation, the membrane was again washed using TBS-T and incubated for a further hour at room temperature with Infrared-labelled goat anti-mouse secondary antibody (LI-COR Inc.). Finally, the membrane was washed using TBS-T, followed by TBS to remove any remaining Tween-20. The Odyssey Clx Imager was used to visualise protein bands. Protein loading was determined using  $\beta$ -actin as a control.

### 2.11. Statistical analysis

Statistical analysis was determined by ANOVA followed by Dunnett's test or Tukey's post-hoc test using Prism 5.0 (GraphPad Inc. San Diego, California, USA). Results are expressed as mean  $\pm$  SE or mean  $\pm$  SD. Statistical significance was  $P < 0.05$ .

## 3. Results

### 3.1. Physicochemical characterisation, degree of hydrolysis and in vitro antioxidant assessment of casein hydrolysates

The different proteolytic preparations produced hydrolysates having distinctly different RP-UPLC (Fig. 1) and GPC (Fig. 2) profiles demonstrating the differing specificities of the proteolytic preparations. The higher specificity of TPCK-Trypsin and pepsin resulted in a lower proportion of low molecular mass (< 500 Da) peptides present in these samples (Fig. 2) and in a lower DH (Table 1). Alcalase®, Flavourzyme® and Prolyve hydrolysis resulted in hydrolysates with the highest proportion of low molecular mass peptides (< 500 Da) and the highest degree of hydrolysis (~14–18%).

All 5 kDa permeates showed significantly ( $P < 0.05$ ) higher antioxidant activity than intact sodium caseinate or the full hydrolysates in both the ORAC and ABTS<sup>+</sup> assays (data not shown). There was no significant ( $P < 0.05$ ) difference in antioxidant activity between the different 5 kDa permeates when measured by the ORAC assay, while the 5 kDa UF permeate of the Flavourzyme® hydrolysate had the highest activity in the ABTS assay (Table 1); significantly ( $P < 0.05$ ) higher than that of the 5 kDa UF permeate of the tryptic hydrolysate. There was no correlation between the DH of the hydrolysates and the antioxidant activity (via ORAC or ABTS<sup>+</sup> assays) of the corresponding 5 kDa UF fractions (Table 1).

*3.2. Effects of casein hydrolysate 5 kDa permeates on cell proliferation in RAW264.7 mouse macrophages, human U937 lymphocytes and human Jurkat T cells.*

RAW264.7 mouse macrophages were exposed to increasing concentrations (0–5% w/v) of the different 5 kDa permeates for 24 h. The MTT assay was then used to assess the effect of each hydrolysate on cell proliferation. Proliferation was generally unaffected by the permeates up to a concentration of 1% (w/v) (Table 2). At a concentration of 5% (w/v), cell proliferation declined significantly ( $P < 0.05$ ) in cells incubated with hydrolysate permeates

generated using Flavorpro Whey, trypsin and pepsin. For Jurkat T cells, the 5 kDa permeates obtained from hydrolysates generated using Promod, trypsin and pepsin significantly ( $P < 0.05$ ) decreased cell proliferation at 5% (w/v), while the Flavourzyme® hydrolysate permeate significantly ( $P < 0.05$ ) increased cell proliferation at this concentration (Table 3). A similar trend was seen with 5 kDa permeates in U937 cells (Table 4). Concentrations of 0.050 and 0.005% (w/v) were, therefore, selected for bioactivity assays involving RAW264.7 cells. Non-cytotoxic concentration of 0.50 and 0.05% (w/v) for Jurkat T cells, and 0.05% (w/v) for U937 were used for bioactivity assays ensuring that all cell viabilities remained greater than 85%.

### 3.3. Antioxidant activity of casein hydrolysate 5 kDa permeates

The comet assay was used to assess the DNA protective effect of the 5 kDa permeates against H<sub>2</sub>O<sub>2</sub>-induced DNA damage in U937 cells. Tail DNA was increased to approximately 80% in U937 cells exposed to H<sub>2</sub>O<sub>2</sub> (80 µmol L<sup>-1</sup>) for 30 min from a control level of approximately 16% in untreated cells. Pre-incubation of U937 cells with the different permeates at 0.05% (w/v) for 24 h did not protect cells against H<sub>2</sub>O<sub>2</sub>-induced DNA damage (Fig. 3).

### 3.4. Cytokine production in Jurkat T cells

Jurkat T cells were stimulated to produce cytokines using 25 µg mL<sup>-1</sup> ConA, following which cytokine (IL-6, IFN-γ, IL-2 and IL-10) production was measured. IL-6 production was seen to decrease in cells exposed to the 5 kDa permeates (Table 5). All hydrolysate permeates at 0.50% (w/v) produced a significant ( $P < 0.05$ ) decrease in IL-6

production compared with control values. No significant ( $P < 0.05$ ) effects were seen in IL-2, IL-10 and IFN- $\gamma$  production after exposure to the 5 kDa permeates at either 0.50 or 0.05% (w/v).

### 3.5. Cytokine production in RAW264.7 cells

RAW264.7 cells were stimulated with LPS at  $0.1 \mu\text{g mL}^{-1}$  or  $2 \mu\text{g mL}^{-1}$  and treated with 5 kDa permeates for 24 h before cytokine analysis (Table 6). At 0.05% (w/v), each of the hydrolysate fractions caused a significant ( $P < 0.05$ ) decrease in IL-6 production compared with cells incubated with LPS alone. At 0.005% (w/v), none of the hydrolysate fractions caused significant ( $P < 0.05$ ) decreases in IL-6 production. Hydrolysate permeates generated using Flavourzyme®, Flavorpro Whey and trypsin caused a significant ( $P < 0.05$ ) decrease in IL-1 $\beta$  production in cells incubated with these samples at 0.050% (w/v). No effect was seen in TNF- $\alpha$  production after treatment with the 5 kDa permeates at any concentration.

### 3.6. NF- $\kappa$ B protein expression in RAW264.7 cells

RAW264.7 cells were stimulated using  $0.1 \mu\text{g mL}^{-1}$  LPS and treated with the 5 kDa permeates at 0.050% (w/v) for 24 h in 60 mm dishes. Cells were then lysed and cell lysates were examined for protein expression of the NF- $\kappa$ B subunit, p65. All hydrolysate fractions caused a significant ( $P < 0.05$ ) decrease in p65 protein expression compared with cells treated with LPS alone (Fig. 4).

## 4. Discussion

It is recognised that utilisation of a variety of analytical techniques is required to capture differing pathways of antioxidant activity. The ORAC assay measures the ability of an antioxidant to prevent peroxy radical oxidation of a fluorescent probe by means of hydrogen atom transfer (HAT). In the ABTS assay, the antioxidant present in the permeate sample scavenges the ABTS radical cation ( $\text{ABTS}^{\bullet+}$ ) by means of electron transfer (Power et al., 2013). In the present study, during initial experimentation it was observed that concentration of the low molecular mass peptides on processing through a UF membrane having a molecular mass cut-off of 5 kDa resulted in an increase in the antioxidant activity as measured by the ORAC assay. However, no further increase in antioxidant activity was achieved on further processing through a 1 kDa membrane (data not shown). Therefore, the 5 kDa permeates of each of the hydrolysates were used for all other determinations.

Studies have reported that casein hydrolysates possess non-cellular antioxidant activity, mainly radical scavenging or electron donating ability. Alcalase® has been frequently used to produce such hydrolysates (Ao & Li, 2013; Chen & Li, 2012; De Gobba, Tompa, & Otte, 2014; Xie, Liu, Wang, & Li, 2014; Xie, Wang, Jiang, Liu, & Li, 2015). Alcalase® was also used in this study; however, no significant difference was seen in the antioxidant activity of this hydrolysate compared with hydrolysates produced by the other enzyme preparations and, interestingly, Flavourzyme® produced a hydrolysate with higher ABTS activity. This may be due to the presence of a larger number of peptides below 500 Da in the Flavourzyme hydrolysate.

No cellular antioxidant activity was observed with the hydrolysates in the present study. Previously, Phelan et al. (2009) reported that non-ultrafiltered casein hydrolysates generated using commercial food-grade enzyme preparations altered glutathione and catalase (CAT) activity in Jurkat T cells, but did not prevent  $\text{H}_2\text{O}_2$ -induced DNA damage in Caco-2



cells. Cermeño et al. (2016) reported that a casein hydrolysate, generated using Prolyve, significantly ( $P < 0.05$ ) protected U937 cells from  $H_2O_2$ -induced DNA damage. The 5 kDa permeate of the Prolyve hydrolysate herein was generated using similar conditions; however, no cell protective effect was seen. Notably, the full hydrolysate was used in Cermeño et al. (2016) while our study used the 5 kDa permeate fraction; this may imply that the cell protective effect was associated with higher molecular mass peptides. Xie et al. (2013) found that casein hydrolysates produced using Alcalase® or the simulated gastrointestinal digestion of casein showed significant protective effects in challenged HepG2 cells by reducing oxidant induced cell death. A follow-on study reported that the Alcalase® hydrolysis of casein produced hydrolysate fractions which enhanced catalase and superoxide dismutase activity and increased viability in  $H_2O_2$ -exposed HepG2 cells. The hydrolysate was fractionated based on charge and negatively charged fractions had greater antioxidant activity (Wang, Xie, & Li, 2016). Results herein indicate that the enzymes used did not affect antioxidant activity as hydrolysate 5 kDa permeates with similar activity were produced in all cases.

In the present study, hydrolysates significantly decreased pro-inflammatory cytokine production (IL-6 and IL-1 $\beta$ ) in T cells and macrophages. Casein hydrolysates, produced using combinations of TGase and Prolyve were previously reported to significantly decrease IL-6 production in Jurkat T cells (Cermeño et al., 2016). Malinowski et al. (2014) also reported that a tryptic hydrolysate of bovine  $\beta$ -casein had significant anti-inflammatory activity in kidney cells. In this case, casein was hydrolysed for 4 h and the hydrolysate 1–5 kDa permeate fractions exhibited significant anti-inflammatory activity in human kidney cells. It was suggested that a group of large hydrophobic peptides were responsible for the anti-inflammatory activity. The hydrolysis of  $\beta$ -casein using cod trypsin has also been reported to have anti-inflammatory activity in kidney cells and larger peptides ( $> 5$  kDa) were reported to have higher activity compared with lower molecular mass peptides (Altmann et al., 2016).

The hydrolysis of sodium caseinate using a bacterial enzyme has also been reported to reduce IL-8 production in TNF- $\alpha$  stimulated Caco-2 cells, as well as downregulating several pro-inflammatory cytokines expression in LPS-stimulated colonic tissue. Activity was reported to be highest in the 1 kDa retentate fraction in this case (Mukhopadhyaya et al., 2014). All the hydrolysates in the present study were 5 kDa permeates and had significant anti-inflammatory activity, particularly those produced using trypsin, Flavourzyme® and Flavorpro Whey.

Two enzyme-only controls were generated based on the high activity of the corresponding hydrolysates and these controls were screened for anti-inflammatory activity in RAW264.7 cells to rule out reagent/enzyme related activity. Enzyme-only controls had little anti-inflammatory activity (data not shown), therefore it was determined that the observed anti-inflammatory activity was due to the hydrolysis of casein by these enzymes. Flavourzyme® has previously been shown to generate hydrolysates from brewer's spent grain that were capable of decreasing IFN- $\gamma$  production in Jurkat T cells (McCarthy et al., 2013a,b). Bamdad, Shin, Suh, Nimalaratne, and Sunwoo (2017) also reported that a Flavourzyme® generated casein hydrolysate decreased nitric oxide production and TNF- $\alpha$  mRNA expression in RAW264.7 cells; however, this hydrolysate was produced using a combined treatment of high hydrostatic pressure and enzymatic digestion. To the best of our knowledge, no other studies have previously reported anti-inflammatory activity with Flavorpro Whey generated casein hydrolysates.

In the present study, while hydrolysate treatment resulted in a decrease in IL-6 and IL-1 $\beta$  production, TNF- $\alpha$  production was unaffected. Yak milk casein hydrolysates, produced using Alcalase®, were reported to decrease the production of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in macrophages (Mao, Cheng, Wang, & Wu, 2011). Hydrolysates used in the study by Mao et al. (2011) had significant in vitro antioxidant activity that may have contributed to the

enhanced anti-inflammatory response; IL-6 and IL-1 $\beta$  production in macrophages was decreased by ~70% and 60%, respectively, in the study by Mao et al. (2011) compared with ~55% and 30%, respectively, in our study. The differing amino acid compositions of milk from different species may also have affected activity, as reported by a study which reported higher antioxidant activity in camel milk casein hydrolysates compared with bovine milk casein hydrolysates (Moslehishad et al., 2013).

The NF- $\kappa$ B pathway is a major transcription pathway in cells linked to inflammation and chronic inflammatory diseases such as atherosclerosis, inflammatory bowel disease and cancer (Yamamoto & Gaynor, 2001). In the current study, the NF- $\kappa$ B subunit p65 (also known as RELA), which is involved in nuclear translocation and activation, was studied after treatment with hydrolysate fractions in LPS-stimulated RAW264.7 cells. The anti-inflammatory activity of all seven hydrolysate 5 kDa permeates may be linked to NF- $\kappa$ B activation as the protein expression of p65 was inhibited in all cases. Milk-derived hydrolysates with anti-inflammatory activity have previously been reported to act through the NF- $\kappa$ B pathway in different cell lines (Altmann et al., 2016; Malinowski et al., 2014; Marcone, Haughton, Simpson, Belton, & FitzGerald, 2015; Nielsen, Theil, Larson, & Parup, 2012). To the best of our knowledge, our study is the first to examine the molecular mechanism behind the anti-inflammatory activity of bovine casein hydrolysates in macrophages. A study of rice protein hydrolysates, prepared using trypsin, yielded similar results to our study, where IL-6 and IL-1 $\beta$  expression in LPS-stimulated RAW264.7 cells were decreased after 24 h hydrolysate treatment and p65 translocation to the nucleus was also decreased (Wen et al., 2016).

## 5. Conclusions

The 5 kDa UF permeates of casein hydrolysates produced using mammalian, plant, fungal and bacterially-derived proteolytic preparations showed significant in vitro anti-inflammatory activity in Jurkat T cells and RAW264.7 macrophages. These hydrolysate permeates had a greater anti-inflammatory effect on IL-6 production in RAW264.7 cells compared with Jurkat T cells. Three of the hydrolysate permeates also significantly decreased IL-1 $\beta$  production in RAW264.7 cells and were produced using either fungal or mammalian derived enzymes. Based on the ability of the hydrolysate 5 kDa permeates to significantly inhibit the expression of the NF- $\kappa$ B subunit, p65, our results also indicate that this anti-inflammatory activity may be dependent on the NF- $\kappa$ B inflammatory pathway. Future studies on the in vivo anti-inflammatory activity of hydrolysate permeates, generated using fungal or mammalian derived enzymes, may be of interest.

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## Figure legends

**Fig. 1.** Reversed phase ultra-performance liquid chromatography profiles of (a) sodium caseinate and (b–h) casein hydrolysate 5 kDa permeates where the enzymatic preparations employed were: (b) Alcalase® 2.4L; (c) Flavourzyme®; (d) Prolyve 1000; (e) Flavorpro Whey; (f) Promod 144MG; (g) Trypsin; (h) Pepsin.

**Fig. 2.** Molecular mass distribution profiles of intact sodium caseinate and 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates generated with different enzymatic preparations using gel permeation chromatography; ■, < 500 Da; ■, 1000–500 Da; ▨, 2000–1000 Da; ▩, 5000–2000 Da; ▪, 10,000–5,000 Da; ▫, > 10,000 Da.

**Fig. 3.** The ability of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) to protect against H<sub>2</sub>O<sub>2</sub> induced DNA damage in U937 lymphocytes; H, cells treated with H<sub>2</sub>O<sub>2</sub> only. Tail DNA damage was measured using the comet assay and expressed as a percentage relative to hydrogen peroxide control values (untreated cells). Data are means ± SD of 2 independent experiments; significance was measured using ANOVA followed by Dunnett's test.

**Fig. 4.** Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.05%, w/v) on NF-κB (p65) protein expression in RAW264.7 cells relative to cells treated with LPS alone (Control, assigned at 100%); p65 protein expression was assessed in LPS stimulated RAW264.7 cells by Western Blot after 24 h sample treatment. The data show one of three independent experiments, which yielded similar results and are the means ± SE of 3 independent experiments; significance was measured using ANOVA followed by Dunnett's test; \* denotes  $P < 0.05$ .

**Table 1**

Oxygen radical absorbance capacity (ORAC) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activities of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates generated with different enzymatic preparations.<sup>a</sup>

Enzyme	Degree of hydrolysis (%)	ORAC value ( $\mu\text{mol TE mg}^{-1}$ FDP)	ABTS value ( $\mu\text{mol TE g}^{-1}$ FDP)
Alcalase®	$18.01 \pm 1.59^a$	$1.072 \pm 0.07^a$	$52.86 \pm 3.46^{a,b}$
Flavourzyme®	$14.43 \pm 0.61^b$	$1.130 \pm 0.076^a$	$71.18 \pm 7.65^a$
Prolyve	$15.65 \pm 0.60^{a,b}$	$1.120 \pm 0.088^a$	$58.38 \pm 3.48^{a,b}$
Flavorpro Whey	$12.86 \pm 0.89^b$	$1.154 \pm 0.007^a$	$67.59 \pm 4.91^{a,b}$
Promod 144MG	$5.23 \pm 0.42^{c,d}$	$1.080 \pm 0.085^a$	$52.14 \pm 2.19^{a,b}$
Trypsin	$7.21 \pm 0.72^c$	$1.080 \pm 0.041^a$	$44.23 \pm 4.83^{b,c}$
Pepsin	$2.31 \pm 0.61^d$	$1.120 \pm 0.044^a$	$59.00 \pm 4.50^{a,b}$

<sup>a</sup> Abbreviations are: TE, Trolox equivalents; FDP, freeze dried powder of hydrolysate. The degree of hydrolysis was calculated for the full hydrolysates prior to ultrafiltration. Data are the mean  $\pm$  SEM of 3 independent experiments; values with different superscript letters are significantly different at  $P < 0.05$  within each assay.

**Table 2**

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) generated using different enzyme preparations on proliferation in RAW264.7 mouse macrophages.<sup>a</sup>

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (% w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	109.8±6.7	116.8±6.5	122.2±7.2	120.0±7.6	87.0±10.5
Flavourzyme®	106.8±5.8	106.7±4.3	111.8±5.7	110.6±4.6	74.0±16.8
Prolyve	107.7±5.5	107.7±7.0	109.5±5.6	109.3±6.1	77.8±13.2
Flavorpro Whey	106.7±9.8	106.6±5.7	103.6±1.2	97.9±1.6	40.4±9.6*
Promod 144MG	99.5±3.1	99.2±4.0	92.0±9.5	90.3±9.3	68.9±13.5
Trypsin	68.7±9.5	64.0±15.7	91.2±5.8	89.9±7.7	59.2±8.7*
Pepsin	107.1±3.5	107.1±6.7	112.3±5.0	110.8±8.2	33.6±6.9*

<sup>a</sup> RAW264.7 mouse macrophages were seeded at a density of  $0.2 \times 10^5$  cells mL<sup>-1</sup> and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test;  $P < 0.05$ ) in cell proliferation, compared with untreated RAW264.7 cells.

**Table 3**

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) on proliferation in human Jurkat T cells. <sup>a</sup>

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (% , w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	89.3±7.5	84.0±10.4	77.9±9.1*	77.2±8.2*	84.3±4.3
Flavourzyme®	91.1±7.5	96.1±1.2	96.1±4.0	117.6±9.1*	242.8±12.4*
Prolyve	96.3±3.8	94.1±4.1	90.9±6.4	94.7±5.1	98.6±5.1
Flavorpro Whey	95.1±2.8	92.7±1.8	90.0±1.6*	101.2±4.8	102.0±7.7
Promod 144MG	98.3±5.9	94.5±5.8	81.6±1.3*	78.8±2.4*	83.8±4.4*
Trypsin	96.9±1.7	90.7±2.6	76.2±9.9*	79.0±3.6*	78.4±5.2*
Pepsin	95.8±7.3	94.6±3.4	78.9±2.8*	77.8±2.4*	13.4±0.5*

<sup>a</sup> Jurkat T cells were seeded at a density of  $1 \times 10^5$  cells mL<sup>-1</sup> and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test;  $P < 0.05$ ) in cell proliferation, compared with untreated human Jurkat T cells.

**Table 4**

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0–5%, w/v) on proliferation in human U937 lymphocytes.<sup>a</sup>

Enzyme	Cell proliferation (% control) with 5 kDa ultrafiltration permeates (% , w/v)				
	0.05	0.10	0.50	1.00	5.00
Alcalase®	102.7±3.3	96.3±4.3	84.9±4.3	84.4±4.4	76.3±9.8*
Flavourzyme®	107.0±7.0	101.2±2.0	94.6±3.6	103.8±10.4	159.4±5.3*
Prolyve	102.8±3.6	97.5±3.5	87.5±2.3	92.4±4.8	91.4±10.4
Flavorpro Whey	95.3±3.1	97.6±1.3	94.3±1.6	94.1±6.8	56.8±6.8*
Promod 144MG	101.5±6.6	89.1±8.3	87.6±1.2	82.5±5.4	71.4±6.3*
Trypsin	99.4±5.0	94.2±2.7	85.8±2.5	80.6 ±5.2	62.8±9.8*
Pepsin	93.7±4.4	94.6±1.9	83.1±3.9*	81.2±3.2*	12.2±0.9*

<sup>a</sup> U937 lymphocytes were seeded at a density of  $1 \times 10^5$  cells mL<sup>-1</sup> and treated with increasing concentrations (0–5%, w/v) of samples for 24 h. Cell proliferation was determined using the MTT assay and values are expressed as a percentage relative to untreated cells. Data are the mean ± SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test;  $P < 0.05$ ) in cell proliferation, compared with untreated human U937 cells.

**Table 5**

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.50 and 0.05%, w/v) on IL-6, IFN- $\gamma$ , IL-2 and IL-10 production in Jurkat T lymphocytes. <sup>a</sup>

Sample	Cytokine production (% control)							
	IL-6		IFN- $\gamma$		IL-2		IL-10	
	0.50%	0.05%	0.50%	0.05%	0.50%	0.05%	0.50%	0.05%
Control	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
Alcalase®	76.7 $\pm$ 2.8*	87.8 $\pm$ 2.9	91.0 $\pm$ 3.9	97.5 $\pm$ 2.8	103.4 $\pm$ 3.8	103.4 $\pm$ 3.8	105.9 $\pm$ 4.0	103.8 $\pm$ 2.9
Flavourzyme®	81.7 $\pm$ 2.1*	87.9 $\pm$ 2.1	89.1 $\pm$ 3.9	94.2 $\pm$ 2.4	101.7 $\pm$ 4.7	101.7 $\pm$ 4.7	111.2 $\pm$ 3.3	104.2 $\pm$ 5.0
Prolyve	79.5 $\pm$ 1.0*	92.8 $\pm$ 4.1	94.2 $\pm$ 3.2	100.3 $\pm$ 1.5	95.0 $\pm$ 5.9	95.0 $\pm$ 5.9	102.7 $\pm$ 5.1	105.2 $\pm$ 0.4
Flavorpro Whey	83.6 $\pm$ 0.8*	92.0 $\pm$ 2.0	103.6 $\pm$ 3.6	100.7 $\pm$ 5.2	103.3 $\pm$ 5.9	103.3 $\pm$ 5.9	106.9 $\pm$ 3.8	107.2 $\pm$ 4.8
Promod 144MG	79.4 $\pm$ 1.5*	99.9 $\pm$ 3.2	93.5 $\pm$ 2.6	103.8 $\pm$ 3.6	100.9 $\pm$ 3.3	100.9 $\pm$ 3.3	111.2 $\pm$ 7.6	108.1 $\pm$ 6.3
Trypsin	80.9 $\pm$ 1.5*	102.7 $\pm$ 4.1	88.8 $\pm$ 2.7	105.7 $\pm$ 3.5	116.7 $\pm$ 8.4	116.7 $\pm$ 8.4	116.0 $\pm$ 6.1	104.3 $\pm$ 9.3
Pepsin	87.2 $\pm$ 2.1*	102.2 $\pm$ 2.6	97.2 $\pm$ 1.4	110.0 $\pm$ 7.6	98.2 $\pm$ 3.2	98.2 $\pm$ 3.2	102.1 $\pm$ 5.0	99.6 $\pm$ 1.2

<sup>a</sup> Jurkat T cells were seeded at a density of  $2 \times 10^5$  cells mL<sup>-1</sup>, stimulated with ConA (25  $\mu$ g mL<sup>-1</sup>) and treated with 0.50 and 0.05% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with ConA alone (control): IL-6 concentration, control = 0.004 ng mL<sup>-1</sup>; IFN- $\gamma$  concentration, control = 0.099 ng mL<sup>-1</sup>; IL-2 concentration, control = 0.147 ng mL<sup>-1</sup>; IL-10 concentration, control = 0.156 ng mL<sup>-1</sup>. Data are the mean  $\pm$  SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test;  $P < 0.05$ ) in cytokine production, compared with Jurkat T cells treated with ConA alone.



**Table 6**

Effect of 5 kDa ultrafiltration permeates of sodium caseinate hydrolysates (0.050 and 0.005%, w/v) on IL-6, IL-1 $\beta$  and TNF- $\alpha$  production in RAW264.7 mouse macrophages.<sup>a</sup>

Sample	Cytokine production (% control)					
	IL-6		IL-1 $\beta$		TNF- $\alpha$	
	0.050%	0.005%	0.050%	0.005%	0.050%	0.005%
Control	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0	100.0 $\pm$ 0.0
Alcalase®	55.4 $\pm$ 7.4*	100.7 $\pm$ 21.1	78.8 $\pm$ 5.3	118.4 $\pm$ 7.8	109.8 $\pm$ 5.1	114.5 $\pm$ 5.2
Flavourzyme®	55.0 $\pm$ 12.4*	82.1 $\pm$ 7.1	74.7 $\pm$ 8.2*	121.4 $\pm$ 9.3	109.6 $\pm$ 4.7	110.4 $\pm$ 3.9
Prolyve	54.2 $\pm$ 3.8*	87.2 $\pm$ 13.3	77.6 $\pm$ 4.1	107.4 $\pm$ 13.9	104.3 $\pm$ 4.8	110.6 $\pm$ 3.3
Flavorpro Whey	47.2 $\pm$ 8.9*	79.5 $\pm$ 4.6	76.4 $\pm$ 3.7*	113.2 $\pm$ 14.5	107.5 $\pm$ 5.1	112.4 $\pm$ 4.7
Promod 144MG	58.4 $\pm$ 12.0*	87.9 $\pm$ 8.1	96.1 $\pm$ 2.9	115.0 $\pm$ 13.0	108.1 $\pm$ 5.6	109.4 $\pm$ 4.0
Trypsin	60.3 $\pm$ 9.8*	99.8 $\pm$ 19.7	74.8 $\pm$ 2.1*	105.1 $\pm$ 16.7	112.4 $\pm$ 6.0	114.0 $\pm$ 4.9
Pepsin	55.7 $\pm$ 4.0*	79.0 $\pm$ 9.7	86.2 $\pm$ 7.2	111.0 $\pm$ 15.6	102.0 $\pm$ 10.8	104.7 $\pm$ 3.4

<sup>a</sup> RAW264.7 mouse macrophages were seeded at a density of  $0.2 \times 10^5$  cells mL<sup>-1</sup>, stimulated with LPS (0.1  $\mu$ g mL<sup>-1</sup> or 2  $\mu$ g mL<sup>-1</sup>) and treated with 0.050 and 0.005% (w/v) 5 kDa UF permeates of sodium caseinate hydrolysates for 24 h. Cytokine production was measured using the enzyme-linked immunosorbent assay (ELISA) and values were expressed as a percentage relative to cells treated with LPS alone (Control): IL-6 concentration, control = 0.219 ng mL<sup>-1</sup>; IL-1 $\beta$  concentration, control = 0.180 ng mL<sup>-1</sup>; TNF- $\alpha$  concentration, control = 0.721 ng mL<sup>-1</sup>. Data are the mean  $\pm$  SE of 3 independent experiments; an asterisk denotes statistically significant difference (ANOVA followed by Dunnett's test;  $P < 0.05$ ) in cytokine production, compared with RAW264.7 mouse macrophages treated with LPS alone.

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ACCEPTED MANUSCRIPT

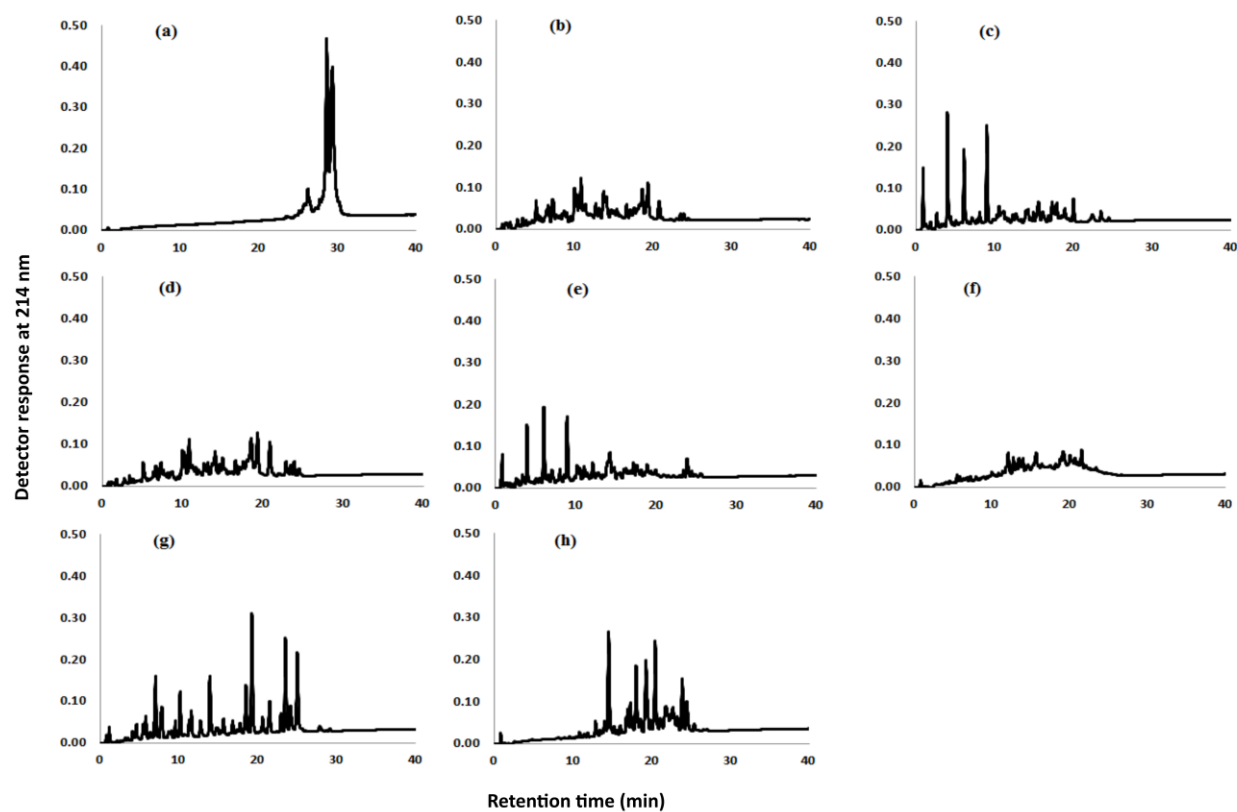


Figure 1:

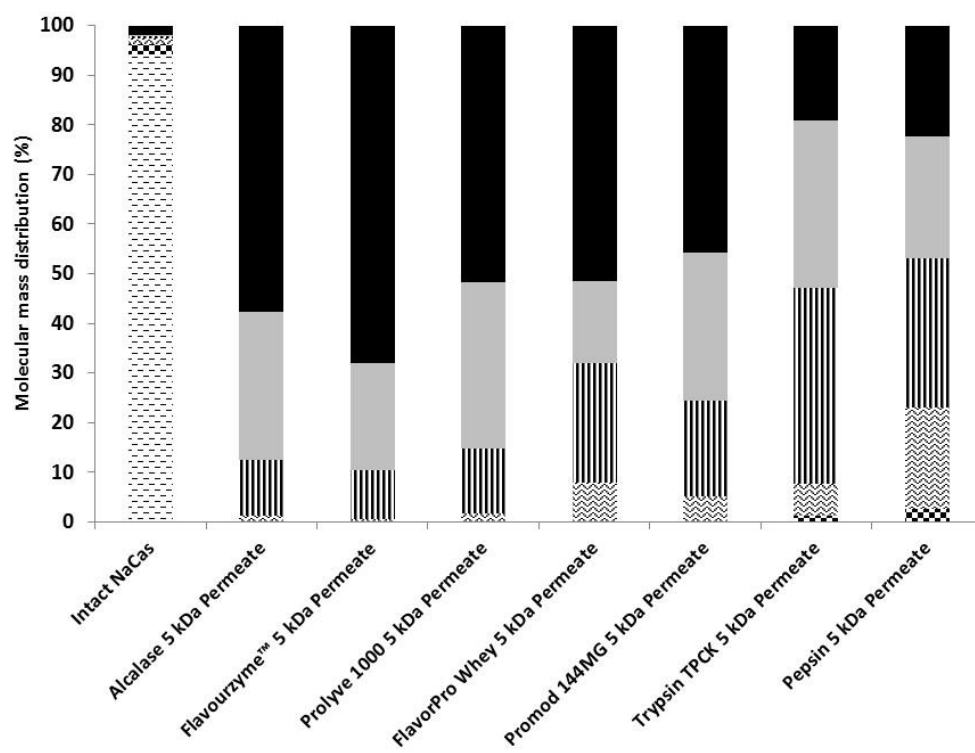
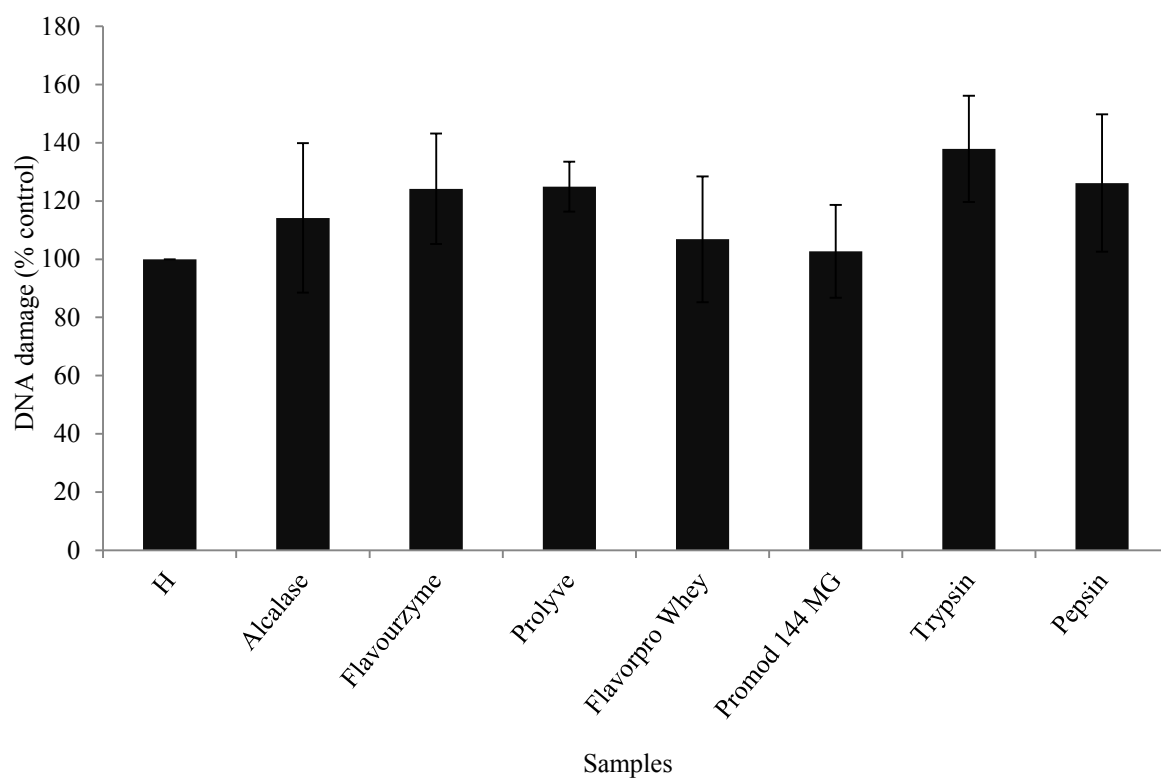
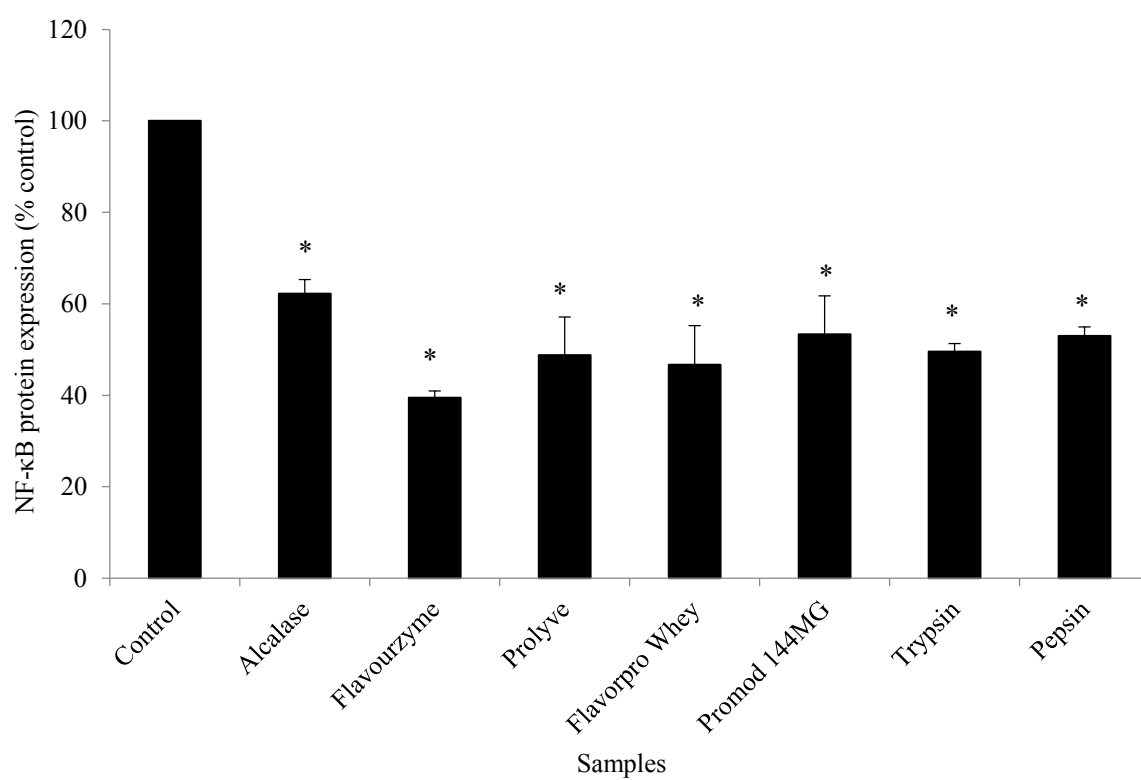


Figure 2:



**Figure 3:**



**Figure 4:**